

IN THE MATTER OF US PATENT APPLICATION
Serial number 09/926,778

VERIFICATION OF TRANSLATION

I, Béatrice ORES,
36 rue de St Pétersbourg
75008 PARIS (FRANCE)

do solemnly and sincerely declare as follows:

1. that I am a qualified Patent Engineer and am well acquainted with both the French and English languages,
2. that the attached document is a true and correct translation into the English language of the specification n° 9907736

filed by COMMISSARIAT A L'ENERGIE ATOMIQUE, with their application for a Patent in France on June 18, 1999

for: "Use of compositions containing soluble forms of HLA-G in the treatment of inflammatory skin pathologies, and method for obtaining them"



Declared by the said

Béatrice ORES

At Paris on June 14, 2006

USE OF COMPOSITIONS CONTAINING SOLUBLE FORMS OF HLA-G
IN THE TREATMENT OF INFLAMMATORY SKIN PATHOLOGIES, AND
METHOD FOR OBTAINING THEM.

5 The present invention relates to the use of
compositions containing soluble forms of HLA-G in the
treatment of skin pathologies, and in particular of
inflammatory dermatoses, to the method for obtaining
said soluble forms of HLA-G and also to the antibodies
10 directed against said soluble forms.

The antigens of the major histocompatibility complex
(MHC) are divided up into several classes, the class I
antigens (HLA-A, HLA-B and HLA-C) which have 3 globular
15 domains ($\alpha 1$, $\alpha 2$ and $\alpha 3$), the $\alpha 3$ domain of which is
associated with $\beta 2$ microglobulin, the class II antigens
(HLA-DP, HLA-DQ and HLA-DR) and the class III antigens
(complement).

20 The class I antigens comprise, besides the
abovementioned antigens, other antigens, termed
unconventional class I antigens, and in particular the
HLA-E, HLA-F and HLA-G antigens; the latter, in
particular is expressed by extravillous trophoblasts of
25 normal human placenta and thymic epithelial cells.

The sequence of the HLA-G gene (HLA-6.0 gene) has been
described by GERAGHTY et al., (Proc. Natl. Acad. Sci.
USA, 1987, 84, 9145-9149); it comprises 4396 base pairs
30 and has an intron/exon organization homologous to that
of the HLA-A, -B and -C genes. More precisely, this
gene comprises 8 exons, 7 introns and a 3' untranslated
end; the 8 exons correspond, respectively, to: exon 1:
signal sequence, exon 2: $\alpha 1$ extracellular domain; exon
35 3: $\alpha 2$ extracellular domain, exon 4: $\alpha 3$ extracellular
domain, exon 5: transmembrane region, exon 6:
cytoplasmic domain I, exon 7: cytoplasmic domain II
(untranslated), exon 8: cytoplasmic domain III

(untranslated) and 3' untranslated region (GERAGHTY et al., mentioned above; ELLIS et al., J. Immunol., 1990, 144, 731-735; KIRSZENBAUM M. et al., *Oncogeny of hematopoiesis, Aplastic anemia* Eds. E. Gluckman, L. Coulombel, Colloque INSERM/John Libbey Eurotext Ltd). However, the HLA-G gene differs from the other class I genes in that the in-frame translation termination codon is located at the second codon of exon 6; consequently the cytoplasmic region of the protein encoded by this HLA-6.0 gene is considerably shorter than that of the cytoplasmic regions of the HLA-A, -B and -C proteins.

These HLA-G antigens are essentially expressed by the cytotrophoblastic cells of the placenta and are considered to play a role in protecting the fetus (lack of rejection by the mother). In addition, in so far as the HLA-G antigen is monomorphic, it may also be involved in the growth or the function of placental cells (KOVATS et al., Science, 1990, 248, 220-223).

Other investigations concerning this unconventional class I antigen (ISHITANI et al., Proc. Natl. Acad. Sci. USA, 1992, 89, 3947-3951) have shown that the primary transcript of the HLA-G gene may be spliced in several ways and produces at least 3 distinct mature MRNAs: the primary HLA-G transcript provides a complete 1200 bp copy (G1), a 900 bp fragment (G2) and a 600 bp fragment (G3).

The G1 transcript does not comprise exon 7 and corresponds to the sequence described by ELLIS et al. (mentioned above), i.e. it encodes a protein which comprises a leader sequence, three external domains, a transmembrane region and a cytoplasmic sequence. G2 mRNA does not comprise exon 3, i.e. it encodes a protein in which the $\alpha 1$ and $\alpha 3$ domains are directly joined; the G3 mRNA does not contain exon 3 or exon 4,

i.e. it encodes a protein in which the $\alpha 1$ domain and the transmembrane sequence are directly joined.

5 The splicing which prevails in order to produce the HLA-G2 antigen leads to the joining of an adenine (A) (originating from the domain encoding $\alpha 1$) with an AC sequence (derived from the domain encoding $\alpha 3$), which leads to the creation of an AAC codon (asparagine) in place of the GAC codon (aspartic acid) encountered at
10 the beginning of the sequence encoding the $\alpha 3$ domain in HLA-G1.

The splicing generated in order to produce HLA-G3 does not lead to the formation of a new codon in the
15 splicing zone.

The authors of that article also analyze the various proteins expressed: the 3 mRNAs are translated into protein in the .221-G cell line.

20

Some of the inventors have shown the existence of other spliced forms of HLA-G mRNA: the HLA-G4 transcript, which does not include exon 4; the HLA-G5 transcript, which includes intron 4, between exons 4 and 5, thus
25 causing a modification of the reading frame when this transcript is translated and, in particular, the appearance of a stop codon after amino acid 21 of intron 4; and the HLA-G6 transcript, which contains intron 4 but which has lost exon 3 (KIRSZENBAUM M. et
30 al., *Proc. Natl. Acad. Sci. USA*, 1994, 91, 4209-4213; European Application EP 0 677 582; KIRSZENBAUM M. et al., *Human Immunol.*, 1995, 43, 237-241; MOREAU P. et al., *Human Immunol.* 1995, 43, 231-236). They have also shown that these various transcripts are expressed in
35 several human fetal and adult cell types, in particular in lymphocytes (KIRSZENBAUM M. et al., *Human Immunol.*, 1995, mentioned above; MOREAU P. et al., *Human Immunol.* 1995, mentioned above).

There are therefore at least 6 different HLA-G mRNAs which potentially encode 6 protein isoforms of HLA-G, 4 of which are membrane-bound (HLA-G1, G2, G3 and G4) and 2 of which are soluble (G5, G6).

5

The immunomodulatory function exercised by these HLA-G molecules has been described and the mechanisms of this function have been elucidated by demonstrating their interaction with lysis-inhibiting receptors present on NK cells (KIR receptors), leading to inhibition of the cytotoxic functions of these cells. Some of the inventors have also shown that certain tumors express HLA-G molecules, which enables them to evade immune surveillance.

10
15 The inventors have now found that soluble forms of HLA-G have a therapeutic action on inflammatory pathological skin conditions.

The expression "inflammatory pathological skin conditions" is intended to mean dermatosis as well as keratinocytes pathologies.

20
25 A subject of the present invention is the use of a composition essentially consisting of at least one soluble form of HLA-G and of at least one pharmaceutically acceptable vehicle (excipient), for preparing a medicinal product for treating inflammatory pathological skin conditions.

30 The excipients combined with said composition are suitable for the desired route of administration; they are selected from excipients known to those skilled in the art.

35 Said soluble HLA-Gs may advantageously be administered generally (orally, parenterally) or locally (topical administration).

In the latter case, said composition is in the form of cream, of lotion, of liposomes or of gel.

Indeed, the Inventors have now found that, unexpectedly, soluble forms of HLA-G being naturally present in psoriasis skin specimens, a composition containing a soluble form of HLA-G is particularly adapted for the treatment of pathological conditions of the skin, particularly due to the major role of T lymphocytes in this disease and due to the capability of HLA-G to inhibit the T functions.

In accordance with the invention, said composition is particularly suitable for treating psoriasis. Psoriasis is a frequent chronic inflammatory disease observed in 2% of individuals of Caucasian populations. A large number of physiopathological studies have made it possible to show that this disease is associated with an infiltration of T lymphocytes, of Th1 subtype, producing interleukine 2 (IL-2) and gamma interferon (IFN γ) (Z. Bata-Csorgo et al. J. Investigative Dermatol., 1995, 89S-94S).

According to an advantageous embodiment of the invention, said soluble form of HLA-G is selected from the group consisting of the soluble isoforms of HLA-G comprising at least one extracellular domain (α 1, α 2 or α 3) and the solubilized forms of HLA-G1, HLA-G2, HLA-G3 or HLA-G4 (membrane-bound isoforms).

Said soluble HLA-Gs comprise at least the α 1 extracellular domain.

Said soluble forms are in particular produced either in a baculovirus, or in *E. coli*.

With regard to the membrane-bound forms, they are advantageously expressed in eukaryotic cells, in accordance with the method described in International

Application PCT WO 98/37098, and then solubilized by treatment of the membrane (stripping agent, such as papain) and suitable purification, for example on an immunoaffinity column with specific antibodies.

5

The term "soluble form of HLA-G" is intended to mean both the soluble HLA-Gs (not comprising a transmembrane domain) and the membrane-bound HLA-Gs which have been solubilized, for example under the conditions specified above.

10

Preferably, said composition, administered topically, comprises between 0.1 and 5 µg/ml, preferably between 0.5 and 2.5 µg/ml, of soluble form of HLA-G.

15

According to another advantageous embodiment of the invention, said soluble form of HLA-G is associated with IL-10; such a composition presents a therapeutical activity particularly significant towards psoriasis.

20

A subject of the present invention is also a method for preparing a soluble HLA-G, characterized in that it comprises the following steps:

25

- coinfecting insect cells with a baculovirus containing the β_2M cDNA and another baculovirus containing the α chain of a soluble isoform of HLA-G;

30

- culturing the transfected insect cells, and

- harvesting the supernatants and purifying the soluble isoform of HLA-G expressed.

35

According to an advantageous embodiment of said method, said soluble isoform of HLA-G is purified using an antibody specific for the soluble isoforms of HLA-G.

According to an advantageous arrangement of this embodiment, said antibody is obtained by immunizing

nonhuman mammals, such as rabbits, with an immunogenic peptide consisting of a 21 amino acid synthetic peptide corresponding to the C-terminal portion encoded by intron 4 of the soluble HLA-G forms, the sequence of which is SKEGDGGIMSVRESRSLSEDL, coupled to the KLH carrier protein.

Besides the above arrangements, the invention also comprises other arrangements, which will emerge from the following description which refers to examples of implementation of the method which is the subject of the present invention and also to the attached drawings, in which:

- figures 1, 2 and 3 illustrate the presence of the various forms of HLA-G in psoriasis.

- figure 4 illustrates the inhibitory activity of the HLA-G isoforms on *natural killer* cells (NK cells) present in peripheral blood; this figure comprises, on the x-axis, the isoform studied and, on the y-axis, the percentage specific lysis. M8 cells (HLA class I⁺, class II⁻ melanoma line cells) transfected either with the vector alone (M8-pCDNA) (Invitrogen) or with the vectors containing the cDNA encoding HLA-G1 (M8-HLA-G1), the cDNA encoding HLA-G2 (M8-HLA-G2), the cDNA encoding HLA-G3 (M8-HLA-G3) or the cDNA encoding HLA-G4 (M8-HLA-G4) are used as targets (T). Peripheral blood mononuclear cells, PBMCs, are used as effector cells (E). The results are expressed as percentage lysis, recorded in 4 h in a chromium 51(⁵¹Cr)-release assay.

- figure 5 illustrates the inhibitory activity of the HLA-G isoforms on a CD8⁺ T lymphocyte line restricted for HLA-A2 presenting a viral peptide originating from the matrix of the influenza virus, positions 58 to 66; this figure comprises, on the x-axis, the isoform studied and on the y-axis, the percentage specific lysis; the effector cell/target cell ratio is 15:1.

It should be clearly understood, however, that these examples are given only by way of illustration of the subject of the invention, for which they in no way
5 constitute a limitation.

EXAMPLE 1 : Production of a soluble form of an isoform of HLA-G in a baculovirus

10 The soluble HLA-G5 isoform, the structure of which consists of 3 extracellular domains, $\alpha 1$, $\alpha 2$ and $\alpha 3$, associated with $\beta 2$ -microglobulin ($\beta 2M$), is derived from an alternative transcript which has a stop codon in intron 4 of the HLA-G gene. The production of a
15 recombinant soluble HLA-G protein makes use of coinfection of the insect cells with a baculovirus containing the complementary DNA of $\beta 2M$, and another baculovirus containing the cDNA of the α chain of HLA-G5. This in fact makes it possible to obtain the
20 physiological form HLA-G5/ $\beta 2M$.

1. Construction of transfer vectors for recombination with the BacTen virus.

25 **a- Insertion of the $\beta 2M$ gene into a transfer vector:**

The $\beta 2M$ coding DNA sequence is inserted into the BgIII-Kpn1 sites of the pTen12 transfer vector (Quantum). The recombinant clone is verified by enzymatic digestion
30 and then amplified and sterilized for the purpose of cotransfection with the DNA of the linearized BacTen baculovirus.

35 **b- Insertion of the gene encoding HLA-G5 into a transfer vector:**

The sequence encoding the HLA-G5 molecule is inserted into the BgIII-Kpn1 sites of the pTen12 transfer vector. The recombinant clone is verified by enzymatic

digestion and then amplified and sterilized for the purpose of cotransfection with the DNA of the linearized BacTen baculovirus (Quantum).

5 2. Construction of recombinant baculoviruses

The first step consists in producing, firstly, HLA-G5A recombinant baculoviruses and, secondly, β 2M recombinant baculoviruses. The two transfer vectors
10 carrying the β 2M and HLA-G5A genes are placed together with linear BacTen in order to cotransfect Sf9 insect cells in culture. The cotransfection supernatants are harvested and lysis plaque cloning is carried out in order to isolate the recombinant baculovirus clones. In
15 order to guarantee the quality of the construct, the DNA of the viral clones is extracted and the insertion of the gene into the correct viral locus is verified by PCR. Six clones were obtained for each of the constructs, and each one was used to reinfect Sf9
20 cells. The culture supernatants and also the cells where then recovered after centrifugation. This protein is purified by immunoaffinity using PAG5-6 antibodies (see Example 4). The structure of the protein is verified by western blot, both with PAG5-6 antibodies
25 (anti-HLA-G5) and BIG6 antibodies (Immunotech) (anti- β 2M) specific, respectively, for the soluble HLA-G5 form and for β 2M. One clone was selected from the 6 clones producing HLA-G5 and one clone was selected from the 4 clones producing β 2M. One HLA-G5 α clone and one
30 β 2M clone were used to coinfect Sf9 cells. It is possible to show, by western blot analysis and immunoprecipitation with a conformational antibody W6/32 (IgG2a, against HLA class I α chains associated with β 2-m (Sigma)), that the supernatant from this
35 coinfection contains the HLA-G5 protein associated with β 2M; it is thus in a form close to the physiological form. These Sf9 cells therefore make it possible to obtain large amounts of soluble HLA-G5 protein.

EXAMPLE 2 : Demonstration of the activity of a composition containing a soluble form of HLA-G on psoriasis. The messenger RNAs are extracted from frozen biopsies of 6 specimens of lesioned psoriatic skin (Pso1-Pso-6, figures 1 and 3) and of 4 specimens of normal skin (healthy skin 1-healthy skin 4, figures 2 and 3), are reverse transcribed, and the cDNA obtained is then amplified by PCR with, on the one hand, primers which recognize all of the HLA-G transcripts [primer G.257 (exon 2) and 3G.U (untranslated 3' end)], used for the PCR amplification of the HLA-G transcripts corresponding to the various known isoforms of HLA-G (figures 1 and 2) and, on the other hand, primers specific for the soluble HLA-G5 sequence, namely primers G.526 and G.i4b (figure 3).

More precisely, the various primers and probes used are, consequently, as follows:

- G.526 (exon 3) and G3.U (3'UT) for the G1, G4 and G5 isoforms;

- G.526 (exon 3) and G.i4b (intron 4) for the G5 isoform;

- G.-3 (partially covering exons 2 and 4) and G3.U (3'UT) for the G2 and G6 isoforms;

- G.3-4 (partially covering exons 2 and 5) and G3.U (3'UT) for the G3 isoform.

The specific HLA-G probes are as follows:

- GR specific for exon 2, and

- G.I4 F (GAGGCATCATGTCTGTTAGG: specific for intron 4), described in P. MOREAU et al., C.R. Acad. Sci. Paris, Sciences de la vie/Life Sciences, 1995; 318; 837-42).

The cDNA from JEG-3 choriocarcinoma cells is used as a positive control for the high levels of HLA-G transcription.

- 5 The specific HLA-G bands are revealed by hybridization with the GR-specific probe located in exon 2. The PCR products, coamplified during the same reaction, with the β -actin primers are detected on the same membrane using a β -actin probe.
- 10 A low level, basal, of HLA-G RNA has been found in 2 out of the 4 normal skin specimens from mammary plasty. It is always related to the specific band of HLA-G1-G5 isoforms. There is no HLA-G RNA in the two other
- 15 healthy skins. In the injured skin biopsies from 6 patients suffering from psoriasis, a higher HLA-G transcriptional level exists, in particular with regard to the G1/G5 isoform (figures 1 and 3).
- 20 In the same way, the signal for the soluble HLA-G5 isoform is absent from the 4 specimens of normal skin (figure 3), but is found in 3 of the 6 individuals exhibiting psoriasis (figure 3).
- 25 An immunohistochemistry study has been performed on cryostat sections, by using two specific antibodies: G1-specific anti-HLA-G 87G antibody, and G6S (GERAGHTY et al. precited), specific of the soluble isoform. In control healthy skin, there is no staining with these
- 30 antibodies. In the injured skin of 6 patients suffering from psoriasis, a staining of the cells of mononucleate infiltrate of the superficial dermis exists with 87G antibody (5/6) as well as 6GS antibody (6/6).
- 35 Cultures of keratinocytes from healthy skin were performed on nutritive layer of irradiated 3T3 fibroblasts. HLA-G expression is absent when measured by flow cytometry on normal keratinocytes, but is inducible by gamma interferon. By RT-PCR, a stimulation

of the level of HLA-G RNA by IL-10, hydrocortisone and gamma interferon is observed.

5 These assays show that HLA-G interferes effectively in the regulation of the T-dependant phenomenons responsible of psoriasis. The results presented here above show that there exists an increased expression of the HLA-G RNA and protein in the psoriasis compared to the healthy skin, and an increased expression of HLA-G
10 protein in the injured skin. This expression seems to come from the cells of the mononucleate infiltrate and is probably due to the infiltrating mononucleate cells. It concerns the predominant HLA-G1 isoform as well as soluble HLA-G5 isoform; these results show the role of
15 this molecule in the totality of the injured site. HLA-G inhibiting *in vitro* the T functions of proliferation and cytotoxicity, its increased presence compared to the healthy skin shows that a composition containing it presents a protective role in psoriasis.

20 In the assays above, the JEG-3 line was cultured in a DMEM medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum, antibiotics and 2 mM L-glutamine. The cell lines do not contain microplasmas.

25 The RT-PCR was performed under the following conditions:

Messenger RNAs were extracted from the precited biopsies with the RNA Now reagent (Biogentex, Inc.)
30 according to the manufacturer's recommendations. The quality of the RNA was verified by electrophoresis on a 1.5% agarose gel under denaturing conditions. cDNAs were prepared starting from 10 µg of total RNA, treated with DNase I (Boehringer Mannheim) using an oligo-
35 (dT)₁₂₋₁₈ primer and the reverse transcriptase M-MLV (Gibco BRL). HLA-G-specific RT-PCR amplifications were performed by using the precited primers (G.257, 3G.U, G.526 and G.i4b).

EXAMPLE 3: Role of the $\alpha 1$ extracellular domain in the inhibitor activity of the HLA-Gs.

The use of cells transfected with each of the HLA-G1,
5 -G2, -G3 or -G4 isoforms as target cells opposite
immunocompetent *natural killer* cells present in
peripheral blood makes it possible to demonstrate that
each of the isoforms is capable of inhibiting the
cytotoxic activity of *natural killer* cells (fig. 4).
10 These experiments were carried out on more than ten
healthy voluntary donors and the significance of the
inhibition exerted by each of the isoforms was
validated with statistical tests (fig. 4).
Similar *in vitro* cytotoxicity assays carried out with
15 the same target cells opposite MHC-restricted CD8+ T
cells specific for an antigenic peptide also
demonstrated that each of the HLA-G1, -G2, -G3 and -G4
isoforms significantly inhibits the cytotoxic activity
of these T cells (fig. 5).

20
Based on the structure of the HLA-G3 isoform consisting
only of the $\alpha 1$ extracellular domain possessing all the
inhibitory properties described above, a conclusion may
be drawn regarding the functionality of this domain.
25 This domain therefore contains the functional motif of
HLA-G and may therefore be used as a pharmacological
agent for the purpose of immunotolerance.

EXAMPLE 4: Production of an antibody, named PAG5-6,
30 which specifically recognizes the soluble forms of HLA-
G (HLA-G5 and HLA-G6), in the form of a polyclonal
serum produced in rabbits.

The immunization of rabbits with an immunogenic peptide
35 consisting of a 21 amino acid synthetic peptide
corresponding to the C-terminal portion encoded by
intron 4 of the soluble HLA-G forms, the sequence of
which is SKEGDGGIMSVRESRSLSEDL, coupled to the KLH
carrier protein, makes it possible to produce a

polyclonal serum which specifically recognizes the soluble forms of HLA-G (HLA-G5 and HLA-G6) via immunoprecipitation techniques, immunoimprinting techniques (Western blot) (fig.6), immunohistochemistry
5 techniques and immunoenzymatic techniques of the ELISA type. The serum is purified on an affinity column (protein G-sepharose) and may be used both for detecting, titrating and purifying the soluble HLA-G forms.

10

As emerges from the above, the invention is in no way limited to its methods of implementation, preparation and application which have just been described more explicitly; on the contrary, it encompasses all the
15 variants thereof which may occur to those skilled in the art, without departing from the context or scope of the present invention.

CLAIMS

1. The use of a composition essentially consisting of at least one soluble form of HLA-G and of at least one pharmaceutically acceptable vehicle, for preparing a medicinal product for treating inflammatory pathological skin conditions.
2. The use as claimed in claim 1, characterized in that said soluble form of HLA-G is selected from the group consisting of the soluble isoforms of HLA-G comprising at least the $\alpha 1$ extracellular domain and the solubilized forms of HLA-G1, HLA-G2, HLA-G3 or HLA-G4.
3. The use as claimed in claim 1 or claim 2, characterized in that said composition comprises between 0.1 and 5 $\mu\text{g/ml}$, preferably between 0.5 and 2.5 $\mu\text{g/ml}$, of soluble form of HLA-G.
4. The use as claimed in any one of claims 1 to 3, characterized in that said composition comprises further IL-10.
5. A method for preparing a soluble HLA-G, characterized in that it comprises the following steps:
 - coinfecting insect cells with a baculovirus containing the $\beta_2\text{M}$ cDNA and another baculovirus containing the α chain of a soluble isoform of HLA-G;
 - culturing the transfected insect cells, and
 - harvesting the supernatants and purifying the soluble isoform of HLA-G expressed.

6. The method as claimed in claim 5, characterized in that said soluble isoform of HLA-G is purified using an antibody specific for the soluble isoforms of HLA-G.
- 5 7. The method as claimed in claim 6, characterized in that said antibody is obtained by immunizing nonhuman mammals, in particular rabbits, with an immunogenic peptide consisting of a 21 amino acids
10 synthetic peptide corresponding to the C-terminal end coded by intron 4 of the soluble forms of HLA-G, whose sequence is SKEGDGGIMSVRESRSLSEDL, coupled to the KLH carrier protein.
- 15 8. A soluble anti-HLA-G antibody, characterized in that it is obtained by immunizing nonhuman mammals, in particular rabbits, with an immunogenic peptide consisting of a 21 amino acid
20 synthetic peptide, whose sequence is SKEGDGGIMSVRESRSLSEDL, coupled to the KLH carrier protein.

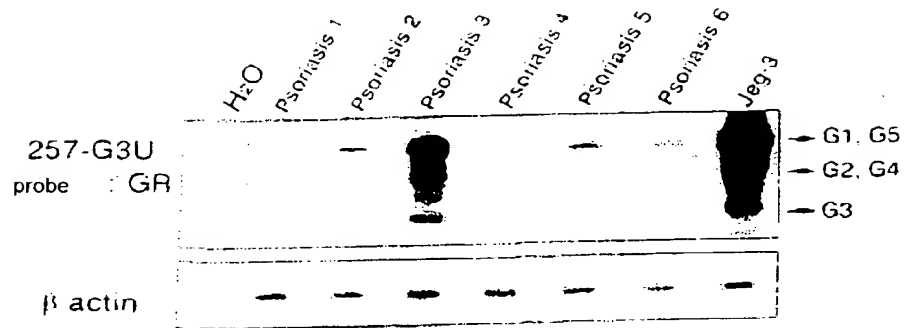


FIGURE 1

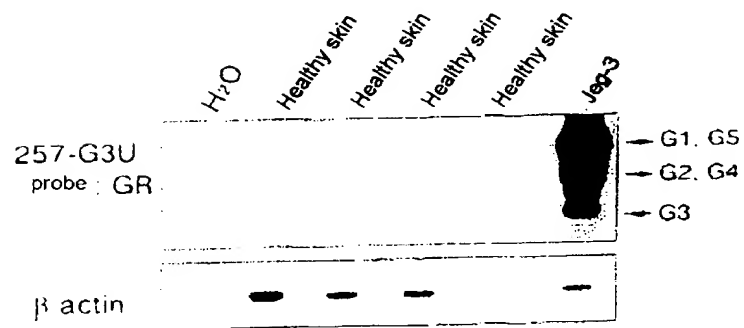


FIGURE 2

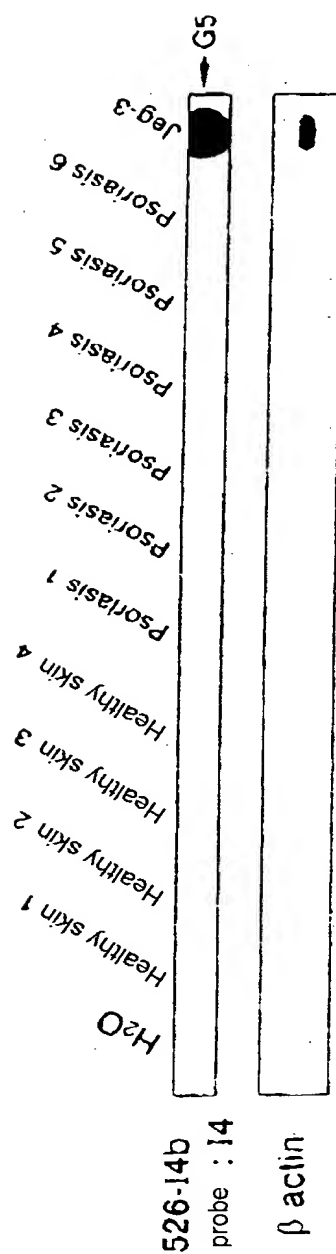


FIGURE 3

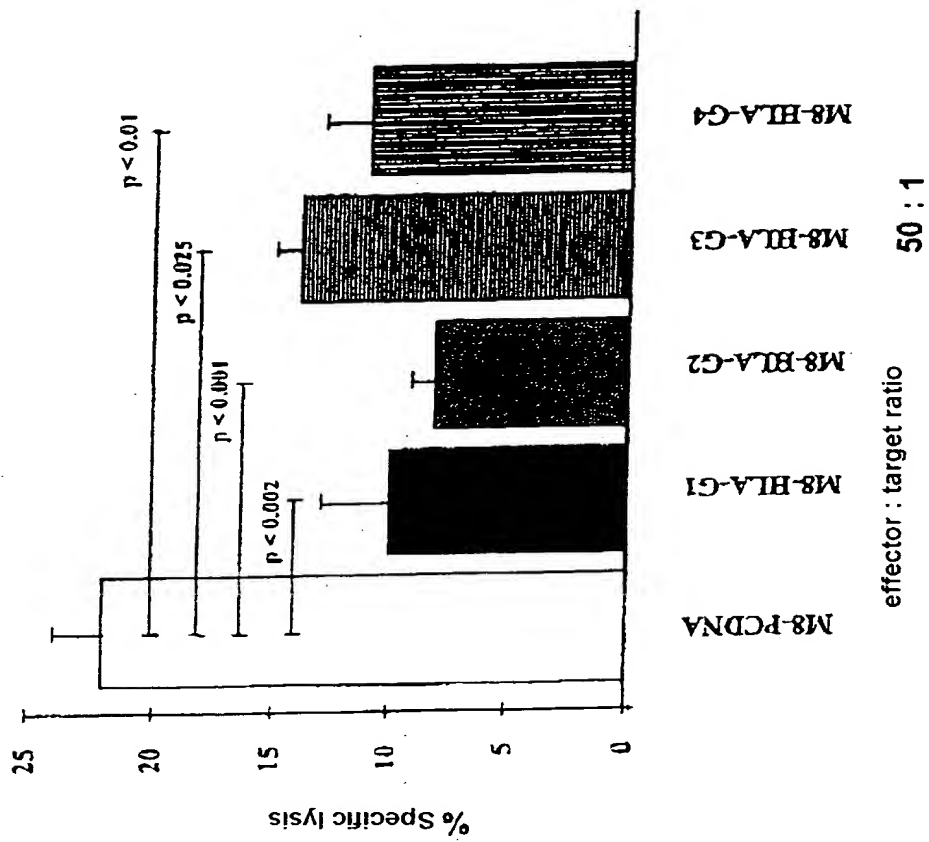
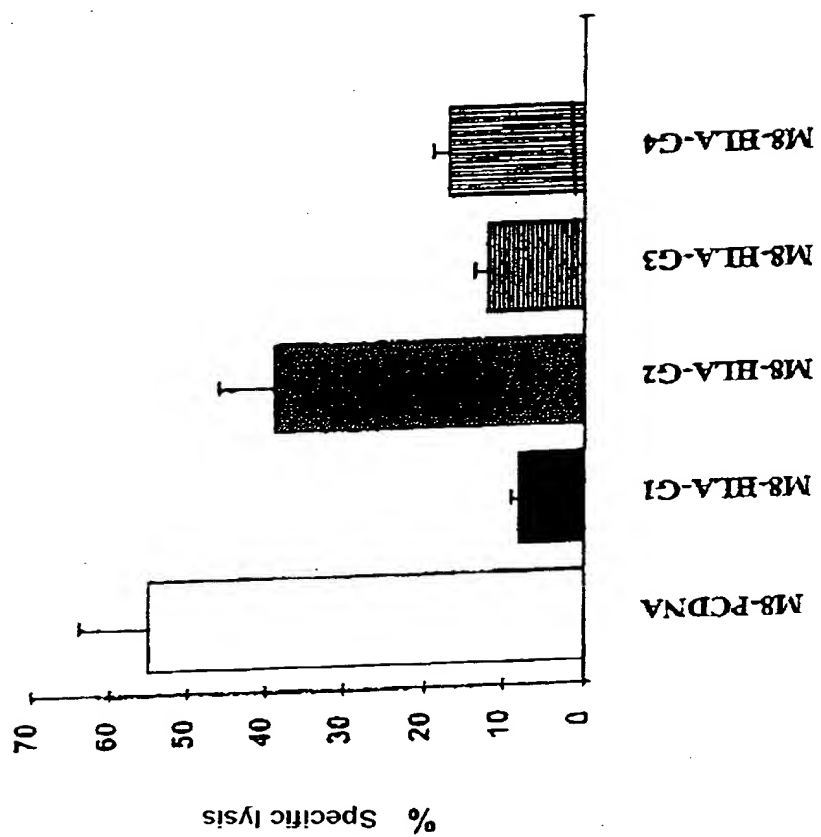


FIGURE 4



effector : target ratio 15 : 1

FIGURE 5